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The role of endothelial cell-bound Jagged1 in Notch3-induced human coronary artery smooth muscle cell differentiation

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ABSTRACT

Phenotype regulation of vascular smooth muscle cells (VSMC) is an important requirement in both tissue engineering and balloon angioplasty strategies. In recent years, it has become increasingly evident that the Notch signalling pathway plays a critical role in regulating vascular morphogenesis during development and the transcription of differentiated VSMC and its maturation. In arteries, Notch3 is the predominant receptor on VSMC and, signalling is initiated upon binding to its ligand, Jagged1. However, little is known on how ligand presenting strategies affect Notch signalling and subsequently upregulation of smooth muscle cell differentiation. In this study, using human coronary artery smooth muscle cells (HCASMC) and human coronary artery endothelial cells (HCAEC), we show several lines of evidence that direct heterocellular cell-cell contact is necessary for VSMC differentiation via Notch3 signalling. First, neither the addition of soluble Jagged1 nor Jagged1 immobilized to protein G beads induced HCASMC differentiation in culture. Second, despite the upregulation of Notch3 expression, EC-conditioned medium failed to induce HCASMC differentiation. However, when HCASMC and HCAEC were co-cultured either on opposite sides of porous membrane or when these cells were co-cultured directly, both Notch3 and VSMC differentiation marker proteins were upregulated. These upregulations were abrogated by Jagged1-specific siRNA. This study provides the first direct evidence that contact of HCASMC and HCAEC is required for regulating smooth muscle cell differentiation. These findings may have clinical importance and therapeutic potential for modulating vascular SMC phenotype during various cardiovascular disease states and in tissue engineering.

1. Introduction

The engineering of vascular tissues incorporating appropriate extracellular matrix components, contractile smooth muscle cells and an endothelial cell monolayer for both clinical and *in vitro* testing applications is highly desirable but the regulation of vascular smooth muscle cells (VSMCs) phenotype in culture has proven to be largely illusive. Unlike cardiac or skeletal muscle cells, VSMCs are not terminally differentiated and are able to continuously modulate their phenotype by expressing unique permutations and combinations of both contractile and synthetic genes in response to changing environmental cues [1]. From a tissue engineering perspective, in the early stages of tissue fabrication, VSMCs are preferred to be in a synthetic phenotype for accelerating cellular proliferation and matrix secretion needed for tissue generation and maturation. Following tissue maturation, VSMCs must switch to a quiescent and contractile phenotype to mimic the functional properties of the native blood vessel. This latter event is largely influenced by the presence of endothelial cells (ECs). Evidence from post-angioplasty follow-ups suggest that, in the absence of ECs, VSMCs acquire a synthetic phenotype leading to extensive migration, proliferation, and matrix synthesis that contribute to restenosis [2]. Therefore, the presence of an intact endothelium slows the proliferation of the underlying VSMC while upregulating the contractile machinery that is a necessary condition for the success of engineered vascular tissues with clinical relevance.

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Within an intact vasculature, specialized cellular extensions from endothelial cells and smooth muscle cells protrude through the internal elastic lamina to interact with each other. Such interaction has been cited as a possible key element in the control of several vascular physiologies and pathologies [3]. Although signal transmission from VSMC to EC is possible, the dominant signals are those transmitted from ECs to VSMCs [3]. Notch signalling is one pathway that plays an important role in the differentiation, maturation, and function of VSMC. The Notch family of receptors, Notch1 to Notch4, are single-pass transmembrane proteins consisting of both an extracellular domain and an intracellular domain (ICD) [4,5]. Upon interaction with the Delta, Serrate/Jagged, Lag-2 (DSL) family of single-pass transmembrane ligands (Jagged1-2 and Delta1, -3, and -4) expressed on neighbouring cells, Notch undergoes proteolytic cleavage, which frees the ICD from the plasma membrane. This results in translocation of the ICD into the nucleus, where it forms a complex with the CSL family of transcriptional repressors (CBF1/RBP-Jk), removing the repression and allowing for downstream target gene (Hes/Hey) transcription [6]. Tissue distribution of the Notch signalling pathway components varies considerably. Notch1, Notch4, Delta1, Delta4, and Jagged2 are expressed in both arterial and venous endothelial cells; whereas, Notch2 expression is restricted to pulmonary endothelial cells [7]. Notch3 is highly expressed in matured arterial smooth muscle cells, where it regulates smooth muscle cell differentiation [8]. The main Notch3 ligand is Jagged1 which is also expressed in arterial endothelial cells. Thus, Notch3 expression in smooth muscle cells is induced upon interaction with endothelial cells. Moreover, Notch3/Jagged1 signalling results in an autoregulatory loop that maintains high levels of Notch3 in smooth muscle cells [9]. This specific pattern of spatial distribution correlates to diverse functions of the Notch family in vascular development and physiology [4,7]. Since all Notch ligands are transmembrane proteins, signalling is often assumed to be mediated by cell-cell contact, indicating a short-range signalling pathway on adjacent cells [10]. In view of this assumption, Jagged1 may require cell-cell contact to activate Notch signalling [11,12]; but, its exogenously added soluble form is reported to induce mesenchymal stem cell differentiation into cardiomyocytes [13], cochlea progenitor cells into primary sensory cells [14], and induces keratinocyte differentiation [15,16]. Soluble Jagged1 was also reported to activate Notch signalling for mediating cell-matrix and cell-cell adhesion [17]. To the contrary, some reports showed that only immobilization of Notch ligands to different substrata activated Notch signalling in epithelial stem cells [18], keratinocytes [19], and hematopoietic cell lines [20,21].

Notwithstanding the above studies on the role of soluble or immobilized Jagged1 to engage in the differentiation of different cells, its role in adult VSMC phenotype modulation is unknown. Understanding the role of Notch3-Jagged1 signalling has implications in vascular tissue engineering and biomaterials design. For example, if soluble Jagged1 is able to engage in Notch signalling, then it can be added to culture media to induce VSMC phenotype modulation via Notch during vascular tissue engineering. If, instead, only surface-immobilized Jagged1 activates Notch signalling, then ligand-functionalized biomaterials/scaffolds can be designed. Towards this goal, we utilized Protein G beads as a model biomaterial surface to gain an insight into biomaterial-mediated Notch3 signalling. Finally, if cell-surface expression of Jagged1 is required for Notch3 signalling, then co-culture conditions can be optimized to determine the time course of phenotype modulation. The objective of this work was, therefore, to investigate the effect of ligand presentation strategies on Notch3 signalling and smooth muscle cell differentiation.

2. Materials and methods

2.1. Cell culture

Primary human coronary artery smooth muscle cells (HCASMC) and primary human coronary artery endothelial cells (HCAEC) were obtained from Lonza (Walkersville, MD) and cultured in SmGM and EGM-2MV (Lonza, Walkersville, MD) respectively. Both media were supplemented with penicillin G (100 U/ml; Invitrogen, Burlington, ON) and streptomycin sulphate (100 μg/ml; Invitrogen). Cells were used between passages 5 and 8. All cultures were maintained in a humidified incubator at 5% CO_2 and 37 °C.

2.2. Co-culture of HCASMC and HCAEC on cell culture inserts

Porous cell culture inserts with 0.4 μm pores (BD Biosciences, Franklin Lakes, NJ) were first coated with 250 μg fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Hank's balanced salt solution (HBSS) for the smooth muscle cell side and with 250 μg growth-factor reduced Matrigel (Becton Dickenson, Franklin Lakes, NJ) diluted in HBSS for the EC side. Matrigel was chosen due to its high laminin content in order to mimic the composition of the *in vivo* basement membrane for which endothelial cells interact and communicate to the underlying smooth muscle cells. For co-culturing both cell types on opposite sides of the inserts, HCASMC were first seeded on the bottom side of the inserts and cultured for 72 h. HCAEC were then seeded on the upper side of the inserts and cultured for an additional 48 h. For direct co-culturing, HCASMC were seeded on the upper side of the insert which was coated with fibronectin. Following 72 h of culture, HCAEC were seeded on top of the HCASMC. In both co-culture scenarios (see schematic drawing in Fig. 6B), the space between the insert and the well was filled by SmGM while EGM-2MV was used inside of the inserts. Media mixing is inevitable but in preliminary screening experiments, we have established that 50/50 SmGM/EGM-2MV had no effect on both cell phenotypes and growth. For protein detection, 6-well inserts were used and $1x10^5$ HCASMC and $1x10^5$ HCAEC were seeded. For immunostaining, 24-well culture plates were used with $2x10^4$ HCASMC and $2x10^4$ HCAEC seeded.

2.3. Immunostaining

For immunostaining purposes, live HCASMC were first stained by 10 μM CellTracker™ Green (Invitrogen, Burlington, ON) for 45 min in serum free growth medium. After incubation with fresh growth medium for another 30 min, cells were trypsinized and the desired amount of cells were seeded on the bottom or top side of the 24-well inserts. After 48 h, cells were co-cultured with HCAEC for another 7 or 21 days. The co-cultured cells were fixed for 10 min at room temperature in 2% paraformaldehyde (EMD Chemicals Inc., Gibbstown, NJ) in divalent cation free phosphate buffered saline (PBS) and permeabilized for 5 min in PBS containing 0.1% Saponin (EMD Chemicals Inc., Gibbstown, NJ). Co-cultures were incubated for 1 h at room temperature in 1% BSA/PBS (Sigma-Aldrich, Oakville, ON) containing Alexa Fluor® 488-conjugated phalloidin (1:100 in PBS) and a VE-cadherin antibody (Santa Cruz Inc, Santa Cruz, CA). Following 3 washes with PBS, cells were incubated for 1 h at room temperature in 1% BSA/PBS (Sigma-Aldrich, Oakville, ON) containing Alexa Fluor® 568-conjugated secondary antibody. Following 3 washes with PBS, nuclei were labeled for 5 min with Hoechst 33342 (10 μg/ml; Sigma-Aldrich) dissolved in PBS. The membranes of the insert were cut out and mounted on glass slides in Vectashield (Vector Laboratories, Burlington, ON) mounting medium covered with a coverslip and sealed with nail enamel. Serial optical sections (0.36 μm thick) were imaged with an LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Toronto, ON) and only selected optical slices are shown.

2.4. Jagged1/Fc protein immobilization to Dynabeads

Protein G Dynabeads (Invitrogen, Burlington, ON) were washed 3 times with PBS (pH 7.4, 0.02% Tween) and mixed with 5 μg of human Jagged1/Fc chimera protein (1277-JG, R&D Systems, Minneapolis, MN) in the original bead volume. The mixture was incubated for 10 min at room temperature on a rotating device and the Jagged1-immobilized beads were washed 3 times with PBS. As a control of Jagged1/Fc chimeric protein, beads were incubated with human IgG solution (5 μg/ml) at the same conditions with those used for Jagged1 binding. This control addresses the effect of the Fc fragment of Jagged1 for any possible non-specific effects of the Fc protein.

2.5. siRNA transfection of Jagged1 in HCAEC

Before transfection, the HCAEC were passaged in growth medium without antibiotics such that they will be 50% confluent in a 10 cm culture dish at the time of transfection. Control siRNA or Jagged1 siRNA (200 pmol; Dharmacon ON-Target PLUS SMART POOL) were mixed with 1 ml Opti-MEM reduced serum medium (Invitrogen, Burlington, ON), then gently mixed with another 1 ml of Opti-MEM reduced serum medium containing 20 μl of RNAiMAX solution (Invitrogen, Burlington, ON), after incubating this mixture at room temperature for 20 min, this complex was added to the culture dish of 50% confluent cells. After 24 h, the siRNA transfected HCAEC were trypsinized and seeded on a plain culture dish or for co-culture with HCASMC. The cells